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# The role of extracellular enzymes in the virulence of the entomopathogenic funfus, Verticillium lecanii, to oat-bird cherry aphid, Ropalosiphum padi (Homoptera:Aphididae)【Research report】

## 蠟蚧幹枝孢菌分解代謝酵素對麥蚜Ropalosiphum padi (同翅目：蚜科)毒力之影響【研究報告】

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## Abstract

The role of extracellular enzymes in the virulence of the entomopathogenic fungus, Verticillium lecanii, to the oat-bird cherry aphid, Ropalosiphum padi, was investigated in the laboratory. Wild type V. lecanii and its UV-induced mutants have varying degrees of reaction when they were cultured on YPDA, gelatin, casein, yeast minimum medium (YMM), and glycerol media. Mutant 1219 showed a negative reaction while 1499 presented a slightly positive reaction to gelatin. Therefore, this is indicated that mutants 1219 and 1499 are protease-deficient mutants. Mutants 1499 and 1519 presented a negative reaction to casein medium. Strong reactions were found with wild type and mutants 1184, 1259, and 1346 grown on gelatin, caesin, YMM, ad glycerol media. Weak reactions were found with 1499 and 1519 grown on YMM and glycerol media. With the API ZYM test, all of the mutants exhibited different reactions to the substrated. In API ZYM, the absence of leucine aminopeptidae was shown on mutants 1499 (Pro-) and 1519 (pro-), and that of acid phosphastase on strain 1519. Wild type, pro+ mutants, and mutants 219(pro-) showed enzymatic activities to all the substrates. In the API CH kit test, wild-type and mutants showed positive reactions to glycerol, 1-O-methyl(a-D-mannoside), 1-O-methyl(a-glucoside), amydalin, soluble starch, glycoge, gentiobiose, l-fucose, and 5-keto-gluconate. Protease(-) mutants showed negative reactions to melezitose. V. lecanii showed a varying degree of development when cultured in aphid cuticle, however, it could not digest grasshopper cuticle. In the bioassy of wild type and mutants of V. lecanii to R. padi, wild type had the shortest LT50 for killing apterae (1.80 d) and alatae (2.02 d), while mutant 1499 had the longest LT50 (3.77 d for apterae; 4.93 d for alatae). As to fecundity, the wild type had significantly lower numbers of progeny for infected apterae (=4.99 progen adult) and alatae(=4.99 progeny/adult). The mutant 1499-infected apterae had significantly higher progeny (8.92 progeny), and mutant 1499-infected alata had 8.84 progeny.

## 摘要

在室內進行分解代謝酵素對蟲生真菌Verticillium lecanii對麥蚜Ropalosiphum padi的毒力生物檢定。當野生型及其紫外線誘變的變種以YPD、明膠、酪蛋白、酵母低量培養基上有不同程度的反應。以明膠培養基培養時變種1219呈負反應而變種1499有微弱的正反應，由此指出二者是解蛋白酵素不足（pro-）的變種。變種1499及1519在酪蛋白培養基培養時呈負反應。而野生型及變種1184、1259及1346在明膠及酪蛋白及YMM和甘油培養時有強烈的反應，而變種1499及1519在YMM及甘油培養基上反應微弱。由以上實驗結果變種1219、1499及1519定為解蛋白質酵素不足之變種。在接著的API ZYM測試中，所有的測試變種的反應皆不同，變種1499及1519對leucine amino-peptidase呈反應，而1519對acidphosphastase呈負反應。野生型、具解蛋白質酵素（pro+）的變種及1219（pro-）對所有的基質皆呈正反應。在API rapid CH測試中，所有的變種包括野生型對甘油、1-O-methyl(α-D-manoside)、1-O-methyl(α-glucoside)、amygdalin、可溶性澱粉、肝醣、gentiobios、L-fucose及5-keto-glu-conate皆呈正反應，而（pro-）變種對Melezitose呈負反應。當V. lecanii無法代謝蝗蟲體皮，而對蚜蟲蛻皮則有不同程度之反應，在對麥蚜的毒性測試時，野生型處理組的LT50最短，無翅型為1.8天，有翅型2.02天，而變種1499則LT50最長，無翅型3.77天、有翅型4.93天。就生殖力而言，野生型處理之有翅及無翅母蟲產4.99隻。變種1499處理之無翅母蟲產8.92隻，有翅母蟲產8.84隻。

**Key words:** Verticillium lecanii, API systems, Ropalosiphu, padi, mutant.
**關鍵詞:** 蠟蚧幹枝孢菌、API ZYM系統、麥蚜、變種。

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# The Role of Extracellular Enzymes in the Virulence of the Entomopathogenic Fungus, *Verticillium lecanii*, to Oat-bird Cherry Aphid, *Ropalosiphum padi* (Homoptera: Aphididae)

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## ABSTRACT

The role of extracellular enzymes in the virulence of the entomopathogenic fungus, *Verticillium lecanii*, to the oat-bird cherry aphid, *Ropalosiphum padi*, was investigated in the laboratory. Wild type *V. lecanii* and its UV-induced mutants have varying degrees of reaction when they were cultured on YPDA, gelatin, casein, yeast minimum medium (YMM), and glycerol media. Mutant 1219 showed a negative reaction while 1499 presented a slightly positive reaction to gelatin. Therefore, this is indicated that mutants 1219 and 1499 are protease-deficient mutants. Mutants 1499 and 1519 presented a negative reaction to casein medium. Strong reactions were found with wild type and mutants 1184, 1259, and 1346 grown on gelatin, caesin, YMM, and glycerol. Weak reactions were found with 1499 and 1519 grown on YMM and glycerol media. With the API ZYM test, all of the mutants exhibited different reactions to the substrates. In API ZYM, the absence of leucine aminopeptidase was shown on mutants 1499 ($pro^-$) and 1519($pro^-$), and that of acid phosphastase on strain 1519. Wild type, $pro^+$ mutants, and mutant 1219($pro^-$) showed enzymatic activities to all the substrates. In the API rapid CH kit test, wild-type and mutants showed positive reactions to glycerol, 1-O-methyl(α-D-mannoside), 1-O-methyl(α-glucoside), amygdalin, soluble starch, glycogen, gentiobiose, L-fucose, and 5-keto-gluconate. Protease(-) mutants showed negative reactions to melezitose. *V. lecanii* showed a varying degree of development when cultured in aphid cuticle; however, it could not digest grasshopper cuticle. In the bioassay of wild type and mutants of *V. lecanii* to *R. padi*, wild type had the shortest $LT_{50}$ for killing apterae (1.80 d) and alatae (2.02 d), while mutant 1499 had the longest $LT_{50}$ (3.77 d for apterae; 4.93 d for alatae). As to fecundity, the wild type had significantly lower numbers of progeny for infected apterae (=4.99 progeny / adult) and alatae (=4.99 progeny / adult). The mutant 1499-infected aptera had significantly higher progeny (8.92 progeny), and mutant 1499- infected alata had 8.84 progeny.

**Key words:** *Verticillium lecanii*, API systems, *Ropalosiphum padi*, mutant.

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## Introduction

Microbial control of insects sparked the interest of the public due to worries about problems of chemical pesticides have caused, as microbial and other non-chemical controls are alternatives for insect pest management. The usefulness of entomothogenic fungi has long been recognized (Gillespie, 1988). The entomopathogenic fungus, *Verticillum lecanii* (Zimmermann) Viegas, has proved to be virulent to the oat-bird cherry aphid, *Ropalosiphum padi*, in previous studies and has the potential as a control agent (Feng *et al.*, 1990; Hsiao *et al.*, 1992). It has long been suspected that entomopathogenic fungi are enabled in this novel mode of infection by the production of cuticle-degrading enzymes (Charnley, 1984). Activity of extracellular enzymes plays an important role in the virulence of *Beauveria bassiana* to lepidopterous insects (Bidochka and Khachatourians, 1990; Gupta *et al.*, 1994). However, the study of enzyme activity was not thorough for *V. lecanii*. Ultraviolet light has been considered a deleterious abiotic factor for most microbial agents when applied in the field. The purpose of this study was aimed to investigate the relationships between extracellular enzyme activity and the virulence to *R. padi* through bioassay of the protease-deficient mutants induced by UV light toward *R. padi*.

## Materials and Methods

### 1. Inoculum preparation

*V. lecanii* (ATCC 46578) was cultured on a yeast-peptone-dextrose agar(YPDA) medium and incubated at 27℃ at photoperiod of 16L:8 D and allowed to grow for 7 d. Then, conidia were harvested and suspended in 0.05% Tween 80 insterile distilled water at a concentration of $10^6$ conidia / ml.

### 2. Preparation of protease-deficient mutants

Conidial suspension was placed on a petri plate (dia. 15 cm) without a lid, and exposed to a UV light source (254 nm) at a 25 cm distance and kept stirred at a moderate speed (Vekpro Stirrer, Scientific Products). Exposure times were 0, 5, 10, 20, 25, 30, 45, 60, 120, 180, 240, and 300 sec. Ten microliters of conidia suspension was taken from the plate at each time interval, pipetted into a YPDA plate, and spread evenly. Each exposure time had 3 replicates. All of the YPDA plates with UV-treated conidia suspension were placed in a 27 ℃, 16L:8D incubator for incubation. After 3 d, the number of colonies (CFU) on each plate was counted and plotted to obtain a survival curve.

From the data of the survival curve, colonies from 30-, 45-, and 60-sec UV exposure were chosen to subculture as our source colonies. Two sizes of colonies were found in our source colonies: large (>2 mm) and small (<2 mm). The small-size colony took 5 d to form a colony, instead of 3 d for the large-size colony.

a. *Obtaining protease-deficient mutants:* Source colonies were subcultured with YPDA medium, 1% casein medium, and 1% gelatin agar medium. After 3 to 5 d, a 2.4% tricholoride acetic acid (TCA) solution was added to each plate to detect the presence of protease. The growth of colonies, and the diameter of the halo zone around the colony were recorded separately with respective medium. Protease-deficient and some weakly protease-producing colonies were subcultured for more than 5 times. Only those with protease-deficient characteristics were chosen as our test materials. After these procedures, 3 protease-deficient mutants (＊1219, 1499, and 1519) and 3 strong protease-producing mutants (＊1184, 1259, and 1346) were chosen as our assay mutants. Autotrophy screening was conducted by the following procedures: 2 different media, yeast minimum medium (YMM) and glycerol medium, were used to characterize *V. lecanii* mutants autotrophy. All these mutants were stripped on the YMM and glycerol plates, and the growth of each mutant was recorded after 7 d.

b. Characterization of mutants: Enzymatic characterization profiles of wild-type strain and 6 mutants were determined using the semi-quantitative API ZYM and API Rapid CH

systems (API laboratory LTD, Quebec, Canada). In API ZYM , 200$\mu$l of spore suspension of each mutant and wild-type at $10^5$ spores / ml was pipetted into the microcupule and incubated for 4 h at 37℃. After incubation, 1 drop each of reagents A and B was added to each microcupule. Five minutes later, the changes of color were recorded and the presence of enzyme reactions was interpreted according to the chart of the API ZYM kit. In API Rapid CH, a 200-$\mu$l spore suspension of wild type and mutants was pipetted into the cupule and tube. After inoculation, the whole tray was placed into a 27 ℃, 16L:8D incubator for 10 d. The presence of spores and mycelia was used as a criterion to determine the enzymatic activities instead of using the rating scale as the kit suggested.

### 3. *Rapalosiphum padi* colony

Barley was planted in non-chemical-amended peat soil. A *R. padi* laboratory cohort was started from approximately 30 apterous adults collected from a Saskatoon, Sask. Canada, wheat field on May 16, 1989 and maintained on barley plants for use. This colony was maintained in an environmental incubator at 27°C with photoperiod of 16L:8D with a 75 W flourescent bulb.

### 4. Grasshopper colony

A non-diapause-strain grasshopper (*Melanoplus sanguinipes*) colony was collected from a Saskatoon wheat field on September 1987 and maintained on barley plants in an insectary room at 28-30℃ with a photoperiod of 16L:8D with a 75 W flourescent bulb.

### 5. Growth of mutants on aphid and grasshopper cuticle

Cuticles of grasshopper and aphid were used as the sole nutrient in this study. Cuticles of aphids that were shed during moulting were collected and ground. Dead grasshoppers were ground and suspended in a 1% Boreaux solution and stirred overnight (TekPro stirrer). On the following morning, the solution was squeezed, decanted, and resuspended in Boreaux solution. These procedures were repeated 3-4 times until the cuticle solution was clear, then the liquid was squeezed, and air-dried, and the cuticle was ground.

Aliquots of 5 mg of aphid cuticle (in 0.5 ml distilled water) and 10 mg of grasshopper cuticle (in 1 ml distilled water) were autoclaved before use. Five microliters of $10^5$ spores / ml spore suspension of wild-type strain and mutants was added to each tube and incubated at 27℃. Ten microliters of spore suspension was added into 10 ml gelatin solution, and incubated in a continuously shaking water bath at 37℃ for 7 d. Spore germination and sporulation of each treatment were observed daily under microscope. The developmental stage was characterized as class I (spore), class II (swollen spore) or class III (budding spore).

### 6. Bioassays

a. Grasshopper: Mutants 1219, 1259, and 1346 and wild-type were used in this bioassay. Ten adult grasshoppers from the laboratory colony (sex ratio 1:1) were dipped into $10^7$ spore / ml suspension of mutants for 5 sec and then placed separately into each container (dia.=4.6 cm, height=7.7 cm) and supplied with 5 pieces of fresh barley grass daily. Daily mortality was recorded for a 1-mo period. Each treatment consisted of 100 grasshopper.

b. Aphid: Newly formed apterous and alata adults of *R. padi* were randomly selected from the stock colony for each bioassay. The bioassay method followed Hsiao *et al.* (1992). Daily mortality and fecundity were recorded. Cadavers were removed and kept in a petri dish, and put in a dessicator at 100% R.H.. The outgrowth of mycelia on the surface of the cadaver was recorded to confirm the causal agent of *V. lecanii*. Analysis of variance, Duncan's multiple range test, and t-test were used to analyze data from each test (SAS, 1985).

## Results and Discussion

### 1. Characterization of mutants

Table 1 indicates the growth and the

protease production of wild type *V. lecanii* and its UV-induced mutants on different media. Mutant 1219 showed a negative reaction while 1499 presented a slightly positive reaction to gelatin, indicating that mutants 1219 and 1499 are protease-deficient mutants. Mutants 1499 and 1519 presented a negative reaction to casein medium while mutants 1259 and 1346 showed a weak reaction. Strong growth was found on wild type and mutants 1184 and 1219, and weak growth was found on 1499 and 1519 when they were grown on gelatin and casein media. The present data indicated that mutants 1219, 1499, and 1519 can be designated as protease-deficient mutants.

In the subsequent API ZYM test, all of the mutants exhibited different reactions to the substrates. The absence of leucine aminopeptidase was shown on mutants 1499 and 1519, and the absence of acid phosphastase on mutant 1519. Wild- type and mutants 1184, 1219, 1259, and 1346 had positive enzymatic activities to all the substrates (Table 2). St. Leger *et al.* (1986) indicated the presence of esterase, esterase lipase, and leucine amonipeptidae in their *V. lecanii* isolates. In the present study, results for wild-type($pro^+$) and mutants 1184, 1259, 1346($pro^+$), and 1219($pro^-$) are consistent with their results except for mutants 1499 and 1519.

The API rapid CH kit allows the study of carbohydrate metabolism of microorganisms. It consists of 49 substrates which may be metabolized by an assimilation or fermentation pathway. Fermentation is shown by a color change due to the aerobic production of acid. Assimilation is indicated by growth of an organism when the substrate is the sole carbon source present.

An API rapid CH kit was used to characterize the carbohydrate utilization of *V. lecanii* mutants in various categories (Table 3). All of the mutants showed positive reaction to glycerol, 1-o-methyl(α-D-

Table 1. Growth and protease production of various UV-induced *V. lecanii* mutants on different media for a 7 days period

| Mutant and wild type | Media | | | | |
|---|---|---|---|---|---|
| | YPD | Gelatin (halo zone dia. mm) | Casein (halo zone dia. mm) | YMM | Glycerol |
| Wild type | +++ | +++(10 mm) | +++(10 mm) | +++[a] | +++ |
| 1184 | +++ | +++(10 mm) | +++(>10 mm) | +++ | +++ |
| 1219 | +++ | − | +++(10 mm) | ++ | ++ |
| 1259 | +++ | ++ (7 mm) | + ( 3 mm) | +++ | +++ |
| 1346 | +++ | ++ (5.5 mm) | + (3.5 mm) | +++ | +++ |
| 1499 | ++ | + (3 mm) | − | + | + |
| 1519 | ++ | ++ (6 mm) | − | + | +/− |

[a] Growth scored or reaction categorized as +weak; ++medium; +++strong.

Table 2. Enzymatic activities of various UV-induced mutants (API ZYM test)

| Enzyme | Mutant | | | |
|---|---|---|---|---|
| | Wild type | 1219 | 1499 | 1519 |
| Esterase | + | + | + | +[a] |
| Esterase lipase | + | + | + | + |
| Leucine aminopeptidase | + | + | - | − |
| Acid phosphastase | + | + | + | − |
| Phosphohydrolase | + | + | + | + |
| N-acetyl-glucosaminidase | + | + | + | + |

[a] Enzymatic activities present (+) or absent (−).

Table 3. Nutrient chacterization of different V. lecanii strains by the API CH method[a]

| Substrate | Enzyme | Mutant | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | wild type | 1184 | 1219 | 1259 | 1346 | 1499 | 1519 |
| 1.Glycerol | Dehydrogenase | m | s | s | m | m | m | s |
| 2.Erythritol | Dehydrogenase | m | m | mm | no | m | s | s |
| 3.D-Arabinose | Dehydrogenase Kinase | m / s | m | s | no | m | m | m |
| 4.L-Arabinose | Dehydrogenase Kinase | no | s / m | no | m | mm | m | m |
| 5.Ribose | Kinase | no | s | s | m | mm | m | mm |
| 6.D-xylose | Isomerase Dehydrogenase | no | mm | no | m | no | s | m |
| 7.L-xylose | Isomerase Dehydrogenase | s | s | s / m | s / m | s / m | s | m |
| 8.Adonitol | Dehydrogenase | m | m | no | m | no | no | m |
| 9.1-O-Methyl-β-D-xyloside | Xylanase | no | mm | m | no | no | mm | b |
| 10.D-galactose | Kinase | m | b | m | mm | mm | s | s |
| 11.D-glucose | Kinase | no | no | no | no | mm | mm | no |
| 12.D-fructose | Kinase | s | mm | no | m | m | mm | no |
| 13.L-mannose | Kinase | s | no | mm | m | m | no | no |
| 14.L-sorbose | Dehydrogenae | no | mm | no | mm | no | b | b |
| 15.L-rhamnose | Isomerase | m | m | m | mm | no | b | s |
| 16.Dulcitol | Dehydrogenase | m | m | no | m | no | mm | s |
| 17.Inositol | Dehydrogenase | no | no | s | s | m | m | m |
| 18.D-mannitol | Dehydrogenase | m | mm | no | m | m | mm | mm |
| 19.D-sorbitol | Dehydrogenase | m | m | no | m | mm | m | mm |
| 20.1-O-methyl (χ-D-manniside) | α-Mannosidase | b | s | mm | m | b | s | s |
| 21.1-O-methyl-χ-D-glucosidase) | α-glucosedase | s / m | b | s / b | s / b | b | s | b |
| 22.N-acetylglucosamine | Kinase | mm | m | s | m | m | mm | b |
| 23.Amygdalin | β-glucosidase | m | b | mm | m | m | mm | s |
| 24.Arbutin | β-glucosidase | no | s | no | no | n | n | s |
| 25.Esculin | β-glucosidase | m | mm | no | no | no | s | mm |
| 26.Salicin | β-glucosidase | no | m | no | no | m | m | m |
| 27.D-Cellobiose | β-glucosidase | no | m | no | no | no | no | m |
| 28.D-Maltose | α-glucosidase | no | s | no | no | m | s | s |
| 29.Lactose | β-galatosidase | m | mm | s | m | no | no | mm |
| 30.Melibiose | α-galactosidase | no | no | no | m | s | s | no |
| 31.Saccharose | β-fructosidase | no | no | no | no | m | mm | no |
| 32.Trehalose | α-glucosidase | no | no | no | no | b | no | no |
| 33.Inulin | Endofructosidase | m | s | m | no | s | b | s |
| 34.Melezitose | α-glucosidase | m | no | no | m | m | no | no |
| 35.Raffinose | α-galactosidase | mm | m | no | no | m | mm | m |
| 36.Soluble starch | amylase | s | mm | s | m | no | m | mm |
| 37.Glycogen | Endoglucanase | s | b | s / m | s / m | m | mm | b |
| 38.Xylitol | dehydrogenase | no | m | s / m | m | b | b | m |
| 39.Geniobiose | β-glucosidase | m | m | m | m | m | mm | m |
| 40.D-Turanose | α-glucosidase | mm | mm | no | s | mm | mm | mm |
| 41.D-Lyxose | Isomerase | m | no | no | m | no | m | no |
| 42.D-Tagatose | Kinase | no | no | no | m | no | b | no |
| 43.D-Fucose | Kinase | no | b | no | no | no | s | b |
| 44.L-Fucose | Kinase | s | s | s | m | b | m | s |
| 45.D-Arabitol | Dehydrogenase | s | b | m | s | b | no | b |
| 46.L-Arabitol | Dehydrogenase | m | b | s | s | b | m | b |
| 47.Gluconate | Kinase | s | m | s | s | b | s | m |
| 48.2-Keto-Gluconate | | s / m | mm | m | no | mm | mm | mm |
| 49.5-Keto-Gluconate | | m | mm | m | s / m | mm | mm | mm |

[a] No=no color change; s=spore; b=budding; m=mycelium; mm=mycelial mass.

manniside), 1-O-methyl(α-glucoside), amygdalin, soluble starch, glycogen, gentiobiose, L-fucose, N-acetyl-D-glucosamine, and 5-keto-gluconate. Protease(-) mutants and mutant 1184 showed a negative reaction to melezitose. Todorova *et al.* (1994) used API Rapid CH to determine chemical profiles of 2 *B. bassiana* strains. The present data suggest that *V. lecanii* might have different enzymatic activities from *B. bassiana*. However, further study of the role of extracellular enzymes is needed.

## 2. The growth of mutants on aphid and grasshopper cuticle

The development of *V. lecanii* in grasshopper and aphid cuticle suspension is shown in Table 4. When conidia was cultured in grasshopper cuticular medium, the conidia did not grow (Table 4) and no mortality was found in the subsequent bioassay toward *M. sanguinipes*. The present data suggests that *V. lecanii* might not be able to utilize grasshopper cuticle, although a positive reaction was shown in wild-type and mutants of *V. lecanii* in the autotrophy screening and API ZYM kit test, which indicates the presence of protease, lipase, and N-acetyl-D-glucosamindase. The cuticle of *M. sanguinipes* is made up of 3 major components: lipids, proteins, and chitin.

Table 4. Development of *V. lecanii* in grasshopper or aphid cuticle suspension

| *V. lecanii* strain and mutants | Time after incubation(d) | On aphid cuticular medium % class categories[a] | | | On grasshopper cuticular medium % class categories | | |
|---|---|---|---|---|---|---|---|
| | | I | II | III | I | II | III |
| Wide type | 1 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| | 2 | 3.0 | 94.0 | 3.0 | 100.0 | 0.0 | 0.0 |
| | 3 | 2.0 | 76.0 | 22.0 | 100.0 | 0.0 | 0.0 |
| | 5 | 1.0 | 53.0 | 46.0 | 100.0 | 0.0 | 0.0 |
| 1184 | 1 | 0.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| | 2 | 35.0 | 65.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| | 3 | 30.0 | 70.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| | 5 | 0.0 | 55.0 | 45.0 | 100.0 | 0.0 | 0.0 |
| 1259 | 1 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| | 2 | 17.0 | 80.0 | 3.0 | 100.0 | 0.0 | 0.0 |
| | 3 | 13.0 | 82.0 | 5.0 | 100.0 | 0.0 | 0.0 |
| | 5 | 7.0 | 62.0 | 31.0 | 100.0 | 0.0 | 0.0 |
| 1346 | 1 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| | 2 | 41.0 | 53.0 | 6.0 | 100.0 | 0.0 | 0.0 |
| | 3 | 21.0 | 73.0 | 6.0 | 100.0 | 0.0 | 0.0 |
| | 5 | 11.0 | 63.0 | 26.0 | 100.0 | 0.0 | 0.0 |
| 1219 | 1 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| | 2 | 36.0 | 60.0 | 4.0 | 100.0 | 0.0 | 0.0 |
| | 3 | 7.0 | 89.0 | 4.0 | 100.0 | 0.0 | 0.0 |
| | 5 | 4.0 | 56.0 | 40.0 | 100.0 | 0.0 | 0.0 |
| 1499 | 1 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| | 2 | 19.0 | 75.0 | 40.0 | 100.0 | 0.0 | 0.0 |
| | 3 | 14.0 | 71.0 | 11.0 | 100.0 | 0.0 | 0.0 |
| | 5 | 7.0 | 72.0 | 21.0 | 100.0 | 0.0 | 0.0 |
| 1519 | 1 | 100.0 | 0.0 | 0.0 | 100.0 | 0.0 | 0.0 |
| | 2 | 15.0 | 80.0 | 5.0 | 100.0 | 0.0 | 0.0 |
| | 3 | 14.0 | 75.0 | 11.0 | 100.0 | 0.0 | 0.0 |
| | 5 | 6.0 | 66.0 | 28.0 | 100.0 | 0.0 | 0.0 |

[a] I=conidiospore; II=swollen conidiospore; III=budding.

About 7.4% of the dry weight of cuticle consists of lipids (Bidochka and Khachatourians, 1994). The lipoidal compounds of *M. sanguinipes* may be mycostatic to entomopathogenic fungus, i.e., *V. lecanii*, or lipids which cover the cuticle may be poorly utilized by *V. lecanii* and thus hinder access to cuticular proteins as was *B. bassiana* when it was cultured with a *M. sanguinipes* cuticular suspension. Various degrees of development were found when *V. lecanii* was cultured on aphid cuticular medium. Most of the conidiospores became enlarged on day 2 (class II) with about 45% started budding on day 5 for wild-type and mutant 1184. For mutants 1259, 1346, 1219, 1499 and 1519, about 21%-30% of conidia starting budding on day 5. It is suggested that in order to digest the cuticle of grasshopper (*M. sanguinipes*), more extracellular enzymes other than protease are needed, or the thickness of different insect cuticles might affect the digestion.

### 3. Bioassay

The value of $LT_{50}$ was used to evaluate the virulence of *V. lecanii* in this study. No mortality was found for the grasshopper bioassay. Wild-type had the shortest $LT_{50}$ for killing apterae (1.80 d) and alatae (2.02 d) aphids while mutant 1499 had the longest $LT_{50}$ (3.77 d for apterae; 4.93 d for alatae) (Table 5). In general, protease-positive mutants had lower $LT_{50}$ values than did the protease-deficient mutants for both morphs except mutant 1814. The difference among the values of $LT_{50}$ of *V. lecanii* might be attributed to the mechanisms of infection, and this needs further study.

The impact of *V. lecanii* on the reproduction of *R. padi* is shown in Table 6. The control had significantly higher numbers of progeny produced by females (apterae=33.58 progeny / female; alatae=16.55 progeny / female), and the wild-type-treated aphids had significantly lower numbers of progeny (=4.99 progeny / female) for both morphs. Slightly lower numbers of progeny were produced by protease(+)-treated apterous adults than numbers of progeny by protease(-) mutants-treated apterous adults. However, significantly lower numbers of progeny were produced when alatae were treated with protease(+) (Table 6) except for mutant 1184. For the comparison of 2 different forms of aphids, a

Table 5. $LT_{50}$ of alatae and apterous adults infected with various mutants of *V. lecanii*

| Strain and mutant | Protease | $LT_{50}$[a] | | | Regression equation[b] | |
|---|---|---|---|---|---|---|
| | | Lower | $LT_{50}$ | Upper | Intercept | Slope |
| | | | Alatae | | | |
| 1346 | + | 2.18 | 2.33 | 2.49 | 6.60 | −4.03 |
| 1259 | + | 2.35 | 2.49 | 2.63 | 7.76 | −5.83 |
| 1184 | + | 2.96 | 3.14 | 3.33 | 5.36 | −2.03 |
| 1519 | + | 2.61 | 2.74 | 2.87 | 8.33 | −6.97 |
| 1219 | − | 3.69 | 3.85 | 4.03 | 7.92 | −7.56 |
| 1499 | − | 4.77 | 4.93 | 5.15 | 7.49 | −7.68 |
| Wild type | + | 1.89 | 2.02 | 2.16 | 8.46 | −6.05 |
| | | | Apterous | | | |
| 1259 | + | 1.81 | 1.88 | 1.95 | 13.86 | −12.66 |
| 1346 | + | 2.80 | 2.98 | 3.18 | 4.99 | −2.36 |
| 1184 | + | 3.14 | 3.28 | 3.42 | 9.02 | −8.67 |
| 1519 | + | 2.99 | 3.14 | 3.30 | 7.32 | −5.97 |
| 1219 | − | 2.84 | 3.11 | 3.96 | 6.92 | −5.91 |
| 1499 | − | 3.59 | 3.77 | 3.96 | 6.92 | −5.91 |
| Wild type | + | 1.75 | 1.80 | 1.86 | 14.13 | −12.75 |

[a] Fiducial limits and time in days (P = 0.05).

[b] Equation of the best-fitting line using the maximum-likehood method.

significant difference was only found in mutant 1259 ($t$=8.4381), and the control ($t$=22.2644). We have no explanation for this data now; therefore further study is needed.

Extracellular chitinase and protease has been suggested to be virulence factors in fungal entomopathogens, i.e., *B. bassiana* and *M. anisopliae* (Bidochka and Khachatourians, 1990, 1994; St. Leger *et al.*, 1996). Inhibition of protease activity or mutants which are deficient in protease production resulted in decreased virulence against insects (Bidochka and Khachatourians, 1990; St. Leger *et al.*, 1988). In the present data, *V. lecanii* wild-type and its mutants 1184, 1259, 1219, and 1346 could induce the above enzymes except for mutants 1499 and 1519 lacked leucine aminopeptidase activity, and mutant 1519 lacked acid phosphastase activity which may be responsible for the higher $LT_{50}$ value for both morphs and higher numbers of progeny for mutants of 1219 and 1499 in the bioassay. Previous studies have indicated that ultraviolet light has deleterious effects on entomopathogens. In the future, we need to consider the UV effect when we apply *V. lecanii* in the field.

Table 6. Fecundity of apterous and alatae *R. padi* infected by different *V. lecanii* strains

| Strain and mutant | Mean no. progeny / female | | $t$ value[b] |
|---|---|---|---|
| | Apterous | Alata | |
| Wild type | 4.99 d[a] | 4.99 e | 0.2596 |
| 1184 | 8.33 bc | 7.23 c | 2.3372* |
| 1259 | 8.05 c | 5.02 e | 8.4381** |
| 1346 | 8.50 bc | 5.25 de | 5.2697* |
| 1519 | 8.38 bc | 5.79 d | 2.55* |
| 1219 | 8.20 c | 7.22 c | 1.6681 |
| 1499 | 8.92 b | 8.84 b | 0.05 |
| Control | 33.48 a | 16.55 a | 22.2644** |

[a] Means followed by the same letter in a given column are not significantly different ($P<0.01$) by Duncan's Multiple Range Test.

[b] *Pair-t* test for the comparison of fecundity bwteen apterous and alata.

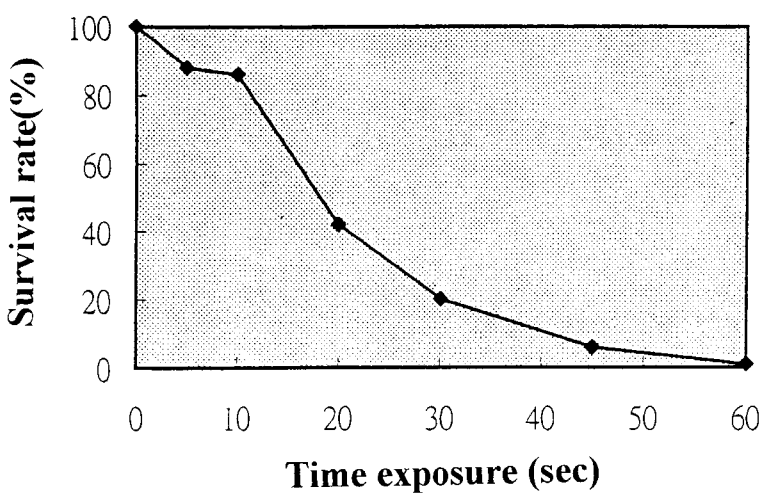


Fig. 1. Survival curve of *Verticillium lecanii* conidia when exposed to ultraviolet light.

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# 蠟蚧幹枝孢菌之分解代謝酵素對麥蚜 *Ropalosiphum padi* (同翅目：蚜科)毒力之影響

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## 摘　要

在室內進行分解代謝酵素對蟲生真菌*Verticillium lecanii*對麥蚜*Ropalosiphum padi*的毒力生物檢定。當野生型及其紫外線誘變的變種以YPD、明膠、酪蛋白、酵母低量培養基上有不同程度的反應。以明膠培養基培養時變種1219呈負反應而變種1499有微弱的正反應，由此指出二者是解蛋白酵素不足（pro-）的變種。變種1499及1519在酪蛋白培養基培養時呈負反應。而野生型及變種1184、1259及1346在明膠及酪蛋白及YMM和甘油培養時有強烈的反應，而變種1499及1519在YMM及甘油培養基上反應微弱。由以上實驗結果將變種1219、1499及1519定為解蛋白質酵素不足之變種。在接著的API ZYM測試中，所有的測試變種的反應皆不同，變種1499及1519對leucine amino-peptidase 呈反應，而1519對acidphosphastase呈負反應。野生型、具解蛋白質酵素（pro+）的變種及1219（$pro^-$）對所有的基質皆呈正反應。在API rapid CH測試中，所有的變種包括野生型對甘油、1-O-methyl(α-D-manoside)、1-O-methyl (α-glucoside)、amygdalin、可溶性澱粉、肝醣、gentiobios、L-fucose及5-keto-gluconate皆呈正反應，而（pro-）變種對Melezitose呈負反應。當*V. lecanii*無法代謝蝗蟲體皮，而對蚜蟲蛻皮則有不同程度之反應，在對麥蚜的毒性測試時，野生型處理組的$LT_{50}$最短，無翅型為1.8天，有翅型2.02天，而變種1499則$LT_{50}$最長，無翅型3.77天、有翅型4.93天。就生殖力而言，野生型處理之有翅及無翅母蟲產4.99隻。變種1499 處理之無翅母蟲產8.92隻，有翅母蟲產8.84隻。

**關鍵詞**：蠟蚧幹枝孢菌、API ZYM系統、麥蚜、變種



*抽印本索取及論文聯繫之負責人